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Regular Article

Differences in thrombus structure and kinetics in patients with type 2 diabetes mellitus after non ST elevation acute coronary syndrome



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ABSTRACT

Introduction: Despite optimal secondary prevention therapy following non-ST elevation acute coronary syndrome (NSTEMI-ACS), recurrent thrombotic events are more frequent in patients with type 2 diabetes mellitus (T2DM).

Materials and Methods: This exploratory study was aimed to evaluate quantitative and qualitative aspects of thrombus. In 28 patients with and without T2DM treated with aspirin and clopidogrel we assessed thrombus quantity using an ex-vivo chamber, platelet reactivity, thrombus ultrastructure and thrombus kinetics one week after NSTEMI-ACS.

Results: T2DM was associated with increased thrombus [14861 (8003 to 30161) vs 8908 (6812 to 11996), μ^2/mm , median (IQR), $p = 0.045$] and platelet reactivity. In addition, diabetic thrombus showed lower visco-elastic tensile strength [(-0.2(-1.7 to 0.7) vs 1.0(-0.9 to 3.3), $p = 0.044$)] and was more resistant to autolysis [(27.8(11.7 to 70.7) vs 78.8(68.5 to 109.6) mm/min, $p = 0.002$)]. On SEM, fibrin fibres in diabetes were thinner, with higher lateral interlinkage and mesh-like organisation. Thrombus quantity correlated inversely with thrombus retraction ($r = -0.450$, $p = 0.016$) but not with platelet reactivity ($r = 0.153$, $p = 0.544$).

Conclusions: Despite optimal antiplatelet therapy, T2DM patients after NSTEMI-ACS developed increased thrombus of lower tensile strength and slower retraction. SEM revealed loosely arranged fibrin fibres. Our data showed significant differences in the magnitude as well as structural and mechanistic characteristics of thrombus in patients with T2DM.

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Abbreviations: ARU, Aspirin reactivity index; CI, Clot index; HDL, High density lipoprotein; HOTR, High on treatment platelet reactivity; LDL, Low density lipoprotein; L parameter, Thrombus lysis parameter; NSTEMI-ACS, Non ST elevation acute coronary syndrome; PRU, Platelet reactivity index; SEM, Scanning electron microscopy; T2DM, Type 2 diabetes mellitus; TEG, Thromboelastography.

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Introduction

Patients with type 2 diabetes mellitus (T2DM) have increased cardiovascular mortality [1,2] due to recurrent thrombotic events [2,3]. In spite of this, current guidelines recommend similar antiplatelet and secondary prevention therapy in non-diabetic and T2DM patients [4,5]. We have shown that blood thrombogenicity remains higher in T2DM after non ST elevation acute coronary syndrome (NSTEMI-ACS) despite treatment with dual antiplatelet therapy [6]. The objective of this exploratory study was to evaluate the impact of T2DM on quantitative and qualitative characteristics of thrombus after NSTEMI-ACS.

Methods

Studies were approved by the Local Research Ethics committee and patients gave written informed consent. Only patients with symptoms of non NSTEMI-ACS, elevated highly sensitive cardiac troponin I ($>0.01 \mu\text{mol/L}$) on 2 occasions, 12 hours apart and proven coronary artery disease (at least one major vessel with $>50\%$ stenosis on coronary angiography) were included. Patients with T2DM were defined as either

a) taking glucose lowering medications, b) HbA_{1c} >6.5% and random blood glucose >11.1 mmol/l or fasting blood glucose >7.0 mmol/l, measured on two occasions (WHO criteria, wholibdoc.who.int/publications/2006/9241594934_eng.pdf, accessed on 25th January 2013). All patients were treated according to current AHA/ESC guidelines on NSTEMI-ACS management including aspirin and clopidogrel [4,5]. Participants underwent whole blood thrombus and platelet reactivity studies one week after NSTEMI-ACS. Thirty consecutive patients were enrolled with 28 patients (14 T2DM) completing all studies. Two patients were excluded because of failure to gain venous access with an 18G cannula.

i) Blood thrombogenicity using the Badimon chamber.

Briefly, the high shear chambers (inner lumen diameter 0.1 mm, Reynolds number 60, shear rate 1690 s⁻¹) mimic the rheologic conditions of a moderately stenosed coronary artery, while the low shear chamber (inner lumen diameter 0.2 mm, Reynolds number 30, shear rate 500 s⁻¹) simulates flow conditions of a normal coronary artery [7]. Surgically dissected porcine aorta served as surrogate for arterial injury and native (non-anticoagulated) blood was perfused for 5 minutes from a large antecubital vein of the patients using 18G cannula at a rate of 10mls min⁻¹. Two high shear and one low shear perfusion studies were performed. After perfusion, aorta specimens were fixed in 10% buffered formalin and stained with modified Masson trichrome. Total thrombus area (μ²/mm) was quantified by planimetry using a Leica DM2000 microscope (Wetzler, GmbH) under 10X magnification and Image ProPlus 4.0 (Media cybernetics, MD, USA) [6,8]. Intra-observer co-efficient of variation (CV) was 4.3%.

ii) Thrombus kinetics.

The visco-elastic properties of thrombus during formation and early phase of autolysis (thrombus retraction) were assessed by thromboelastography (TEG®; Haemonetics Corp, MA, USA). The time to form stable thrombus (R-time), maximum amplitude of thrombus formation (MA), K (kinetics of the rate of thrombus formation) and α (angle between R and K) were recorded. The overall clot index (global measure of thrombus strength) was calculated (clot index = 0.6516R - 0.3772 K + 0.1224MA + 0.0759α - 7.7922). Thrombus retraction was defined as the rate of decline in thrombus strength from its maximum amplitude, measured over 90 minutes (L parameter, mm/min) [9,10].

iii) Platelet reactivity.

Whole blood platelet reactivity was measured by VerifyNow® (Accumetrics, CA, USA) using arachidonic acid reactive units (ARU) and P2Y₁₂ reactive units (PRU). PRUz are measured after stimulation with adenosine diphosphate and PRUb after stimulation with thrombin. We calculated percentage P2Y₁₂ inhibition (100X (PRUz-PRUb)/PRUb). High on treatment platelet reactivity (HOTR) to aspirin was defined as >500 ARU and to clopidogrel as >240 PRUz [11,12].

iv) Systemic biomarkers.

Blood samples were stored at -80C after centrifuging at 1550 g for 5 minutes and thawed immediately before analysis of P-Selectin and soluble CD40 ligand (R&D systems, UK) using sandwich ELISA assays. Full blood count, fibrinogen, lipid levels and HbA_{1c} (DCC aligned) were also analysed.

v) Scanning electron microscopy.

Thrombus generated in the chamber was preserved in 2% glutaraldehyde, and then dehydrated in graded ethanol, critical-point dried and gold-coated. Ultrastructural analysis was performed using an S240 SEM (Cambridge Instruments, UK) on four randomly chosen thrombus samples (2 in each group).

vi) Statistical methods.

Statistics Data is shown as median and interquartile range for uniformity. Differences between groups were analysed by Mann

Whitney U test and Spearman rho test were used to test correlation. Categorical data were analysed using chi square test. As there were no published data to base power calculations, we chose a sample size of 30 based on literature evaluating antiplatelet therapy in T2DM using the TEG assay [13].

Results

Baseline characteristics are presented in Table 1. By virtue of selection, T2DM patients demonstrated the typical metabolic syndrome phenotype with higher triglycerides, HbA_{1c} and systolic BP while all other parameters were similar with the exception of lower LDL cholesterol in T2DM.

Thrombus Quantification

T2DM was associated with increased thrombus in both high shear and low shear conditions in the ex-vivo chamber. At high shear rate, thrombus area (μ²/mm, interquartile range), was 14861 (8003–30161) vs 8908 (6812–11996) for T2DM and control groups, respectively [p = 0.045] and low shear thrombus was 10715 (6562 – 15932) v 6062 (3865–6312) [p = 0.007]. An association was seen between plasma fibrinogen and thrombus in the combined cohort (r 0.551, p = 0.002) (Fig. 2). There were no significant correlations between thrombus and HbA_{1c}, LDL cholesterol, body mass index and diabetes duration.

Thrombus Kinetics on Thromboelastography

The time to form stable thrombus (marker of initiation of fibrin polymerisation) was prolonged in T2DM whilst clot index (CI, measure of the thrombus strength) was lower [median (inter quartile range), -0.2(-1.7 to 0.7) vs 1.0(-0.9 to 3.3),

Table 1
Baseline characteristics.

Median (interquartile range) or %[n]	T2DM (n = 14)	Non T2DM (n = 14)	P value
Demographic data:			
Age, years	62 (51–68)	54 (51–63)	0.401
Male gender,	71.4 [10]	92.9 [13]	0.331
Body mass index, kg/m ²	33.4(29.3–43.4)	30.6 (27.4–30.7)	0.114
Waist to hip ratio	1.0(0.9–1.0)	1.0(0.9–1.0)	0.781
Systolic BP, mmHg	134 (124–147)	120 (112–136)	0.031
Diastolic BP, mmHg	77(72–82)	72 (68–80)	0.227
Risk profile % (n):			
Hypertension	64.3 [9]	21.4 [3]	0.052
Chronic Kidney Disease	21.4 [3]	7.1 [1]	0.601
Medications:			
B blockers	85.7 [12]	100 [14]	0.483
Calcium channel blockers	28.6 [4]	7.1 [1]	0.331
Laboratory data:			
HbA _{1c} , %	6.6 (6.2–8.2)	5.6 (5.3–6.1)	–
Random plasma glucose, mmol/l	8.2 (6.3–11.4)	6.9 (4.9–7.8)	–
Fasting plasma glucose, mmol/l	7.3 (5.6–10.9)	5.1 (4.6–5.5)	–
Haemoglobin, g/dl	13.8 (12.8–15.4)	13.7 (12.9–15.3)	0.865
Platelets, x1000/mm ³	252 (191–309)	257 (209–354)	0.448
Fibrinogen, g/l	4.4 (4.1–4.6)	3.6 (2.9–4.4)	0.095
Creatinine, μmol/l	100(86–110)	99 (81–106)	0.6030
Demographic data:			
Total cholesterol, mmol/l	3.2 (2.6–4.4)	3.4 (3.0–4.0)	0.667
LDLc, mmol/l	1.3 (1.0–1.7)	1.9 (1.6–2.5)	0.023
HDLc, mmol/l	0.9 (0.7–1.1)	0.9 (0.8–1.2)	0.769
Triglyceride, mmol/l	1.8 (1.4–3.4)	1.1 (0.9–1.5)	0.002
Troponin I, μg/l	1.2 (0.1–10.1)	2.0 (1.1–4.1)	0.408

Table 2
Thromboelastography and platelet reactivity indices.

Median (interquartile range)	T2DM (n = 14)	Non T2DM (n = 14)	P value
<i>Thrombus kinetics:</i>			
R time, min	7.3 (6.6 to 8.5)	6.6 (4.6 to 7.1)	0.021
Maximum amplitude, MA, mm	67.7 (62.0 to 70.1)	67.4 (63.8 to 74.1)	0.573
K time, min	1.8 (1.7 to 2.2)	1.6 (1.2 to 2.3)	0.476
α -angle	64.5 (54.5 to 73.1)	68.6 (58.0 to 66.8)	0.306
Clot index	-0.2 (-1.7 to 0.7)	1.0 (-0.9 to 3.3)	0.044
Rate of thrombus retraction (L) mm/min	27.8 (11.7 to 70.7)	78.8 (68.5 to 109.6)	0.002
<i>Platelet Reactivity</i>			
ARU	419 (398–422)	403 (390–404)	0.623
PRUz	259 (222–311)	205 (189–232)	0.028
Percentage inhibition, %	20 (9–32)	32 (22–34)	0.083
P selectin, $\mu\text{g/ml}$	74.8 (59.3 – 77.0)	53.1 (39.7 – 68.9)	0.062
CD40 ligand, $\mu\text{g/ml}$	2934 (2046–3520)	3512 (1674–4220)	0.711

R time: Time to form a stable thrombus (min).

Maximum amplitude is the maximum strength of the formed thrombus (mm).

K time: time taken for blood to achieve 20 mm amplitude of thrombus (min).

α angle: Angle between R and the first 20 mm of amplitude tracing.

Both K value and α angle represent the speed of thrombus formation.

Clot index is a dimensionless value, representing overall thrombus strength and is derived from the formula: $0.6516R - 0.3772K + 0.1224MA + 0.0759\alpha - 7.7922$.

Rate of thrombus retraction (L parameter) represents early phase of autolysis and is measured from the rate of decline in thrombus strength after it has reached the peak value, MA.

ARU: arachidonic acid reactive units.

PRUz: P2Y12 reactive units after stimulation with adenosine diphosphate.

Percentage inhibition: $100X(\text{PRUz}-\text{PRUb}/\text{PRUb})$.

$p = 0.044$) (Table 2). The rate of thrombus retraction, over 90 minutes (L parameter) in T2DM was slower, mm/min, [mm/min, median (inter quartile range), 27.8(11.7 – 70.7) vs 78.8(68.5-109.6) $p = 0.002$]. All other TEG parameters were similar between groups (Table 2). Thrombus retraction or

autolysis correlated negatively with thrombus area ($r = -0.450$, $p = 0.016$). The maximum amplitude of the thrombus (MA) upon stimulation with kaolin correlated to platelet count ($r = 0.576$, $p = 0.002$) and there were no correlations between other TEG parameters and platelet reactivity indices.

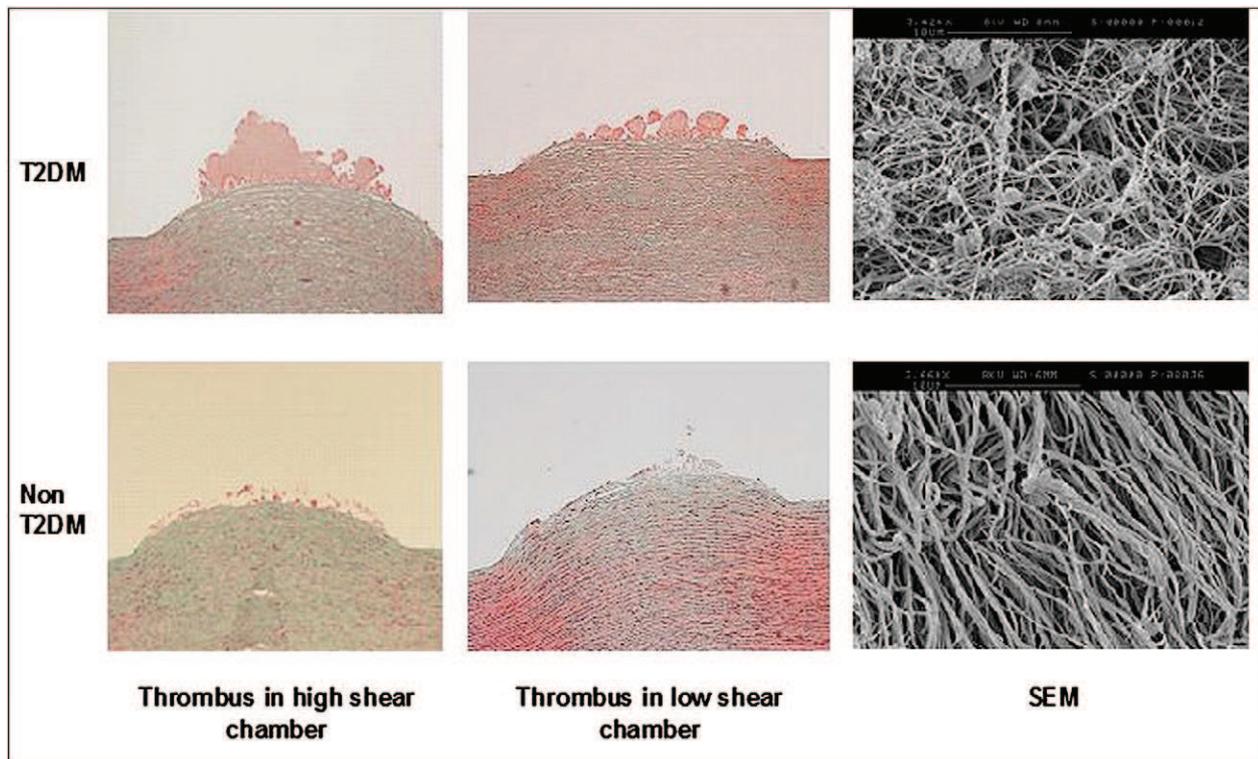


Fig. 1. Thrombus of patients from Badimon chamber (left and middle column) after staining with modified Mason trichrome. Platelet rich thrombus was stained in pink with the tunica media of the aortic tissue stained in green. The right column shows the representative images of the thrombus under scanning electron microscopy (SEM) at 3.4×10^3 times magnification. Diabetic thrombus was increased in quantity occupying a larger surface area over the tunica media. The SEM appearance showed loosely woven thrombus with thinner fibrin fibres and mesh like appearance with less twisted arrangement compared to those without diabetes.

Platelet Reactivity

Aspirin reactive units (ARU) were similar between groups. However, P2Y12 reactive units were higher in T2DM [PRUZ: median (IQR) 259 (222–311) vs 205 (189–232), $p = 0.028$]. One patient (with T2DM) had HOTR to aspirin whereas 13 patients [9 (64.2 %) with T2DM and 4 (28.6 %) without had HOTR to clopidogrel. The biochemical markers of platelet reactivity, P selectin and sCD40 were similar between groups (Table 2). There was no correlation between PRUZ values and thrombus area ($\rho = 0.153$, $p = 0.544$).

Thrombus Structure on SEM

In non-T2DM, thrombus appeared organised with thick fibrin fibres arranged longitudinally in one direction, and few side branches. In contrast, in patients with T2DM, thrombus appeared disorganised with thinner fibrin fibres displaying more side-branches, giving a tangled, mesh-like appearance (Fig. 1). The differences in thrombus structure are displayed in Table 3.

Discussion

In this pilot study, we describe the impact of T2DM on the quantity as well as the structural and mechanical characteristics of thrombus after NSTE-ACS. Thromboelastography (TEG®) [9,13], showed that the visco-elastic property of whole blood and thrombus strength (fibrin-

platelet binding) during thrombogenesis and autolysis was different in T2DM.

We observed that thrombus in T2DM was larger and more stable, but slower to undergo lysis. Studies undertaken with the perfusion chamber revealed increased thrombus both at high and low local shear rate conditions in T2DM patients. The most striking difference in thrombus kinetics was an approximately three fold slower rate of clot retraction in diabetic participants. The time to form stable clot was also increased whilst clot index (a measure of strength) was decreased. Of interest, the observations on the different structural and kinetic characteristics of the thrombus have not been previously reported in whole blood testing. The rate of thrombus retraction was negatively correlated to thrombus quantity in this study which suggests that thrombus, in addition to being higher in quantity, also persists longer after initial formation. In vivo, persistence of thrombus would provide a nidus for more thrombus formation and this may lead to a pathophysiological cycle of ‘thrombus begetting thrombus’ in patients with T2DM. Although in-vitro studies show impaired fibrinolysis (later phase of autolysis) in T2DM [14], little is known of thrombus retraction which represents the early phase of autolysis.

SEM analysis of the thrombus (qualitative analysis) as an exploratory pilot study illustrated some important ultrastructural characteristics further explaining these findings. In the early stages of thrombogenesis (5 minutes of Badimon chamber study), thrombus in T2DM was composed of less compact and loosely arranged fibrin to fibrin structure. Scanning electron microscopy of thrombus revealed a markedly different architecture between the diabetic and non-diabetic patients. Earlier studies using reconstituted plasma have shown altered fibrin structure in patients with T2DM, with reduced elasticity [15]. Alterations in fibrin structure in patients with T2DM have been attributed to non enzymatic glycation of fibrinogen. Glycated fibrinogen enhances thrombogenicity by increasing fibrin polymerisation and cross-linking, reducing tissue plasminogen activator and plasminogen binding, and slowing plasminogen to plasmin conversion [16]. This arrangement of the fibrin fibres was similar to that seen in our study using whole blood in a surrogate model of plaque rupture.

Findings from thromboelastography and SEM are complementary and the differing ultrastructural characteristics may in part explain the differences in thrombus kinetics seen on TEG®. Thrombus in T2DM had thinner fibrin fibres, with more lateral aggregations, occurring in a disorganised, tangled or web-like fashion. This may favour propagation of thrombus by trapping more cellular elements, and in addition, may make clot retraction and degradation more difficult. Thus the increased thrombus quantity in T2DM may be due to both increased blood thrombogenicity generating larger thrombus and decreased degradation. The presence of thinner fibrin fibres in T2DM may result in lower visco-elastic strength of diabetic thrombus and could be one explanation for the differences seen on TEG® [17]. The finding of loosely bound but persistent thrombus in diabetes may increase the potential for embolisation to distal arteries resulting in increased end organ damage. In addition, a less compact and loosely bound fibrin fibre arrangement is a marker of poor fibrinolysis [18]. Presence of less longitudinal twisting in T2DM could result in lower visco elastic strength of diabetic thrombus and can thus explain the differences seen in TEG® studies [19].

T2DM is a prothrombotic state associated with HOTR [14,20]. More patients with T2DM had HOTR with VerifyNow®, findings similar to the main study. More patients with T2DM had HOTR consistent with earlier reports [21,22]. In our small cohort of patients on optimal secondary prevention therapy there were no differences in other indirect measures of platelet reactivity (plasma fibrinogen, P selectin and soluble CD40 ligand) in those with and without diabetes. Platelet counts showed an association to MA in our study implying a significant contribution of platelets to the viscoelastic property of thrombus. Again, we did not observe any correlation between VerifyNow® indices and thrombus area, a finding we have previously reported in a population

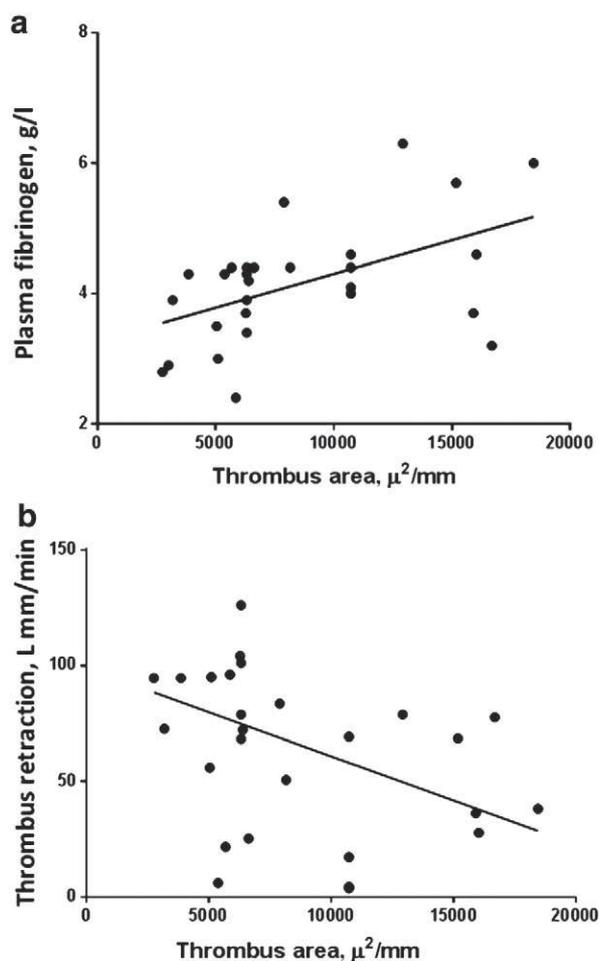


Fig. 2. Correlation graphs between thrombus area and various parameters. Correlation analysis: a. Serum fibrinogen correlated with thrombus area, $r = 0.551$, $p = 0.002$. b. Thrombus retraction or autolysis correlated with thrombus area, $r = -0.450$, $p = 0.016$.

Table 3
Differences in thrombus structure between patients with and without type 2 diabetes mellitus (T2DM) after non ST elevation acute coronary syndrome (NSTEMI-ACS).

T2DM after NSTEMI-ACS	Non DM after NSTEMI-ACS
Quantitatively, larger thrombus generation	Less thrombus formation
Thrombus had lower viscoelastic strength and this could make diabetic thrombus easier to undergo embolization (“friable thrombus”)	Higher viscoelastic strength resulted in “compact thrombus” and resistance to micro embolisation
Thrombus was slower to undergo autolysis and thus could persist (both as occlusive and non occlusive thrombus) in coronary vasculature longer	Autolysis of the thrombus was not delayed and this would favour early clearance of thrombus
Persistence of thrombus in T2DM may result in further propagation as “thrombus begets thrombus”	Faster clot retraction would protect against propagation of thrombus
Ultrastructure of diabetic thrombus showed thinner fibrin fibres	Ultrastructure of the thrombus showed thicker fibrin fibres which were arranged parallel and twisted around the axis
Larger numbers of fibrin fibres were arranged in a mesh or sieve like fashion	Densely arranged fibrin appeared in a more organised fashion with fewer side branches

with stable coronary artery disease [6]. The standard TEG indices did not show any correlation to thrombus either. The negative finding of unaltered kaolin stimulated TEG® parameters (standard TEG®) after aspirin and clopidogrel therapy is not surprising. Kaolin stimulates thrombogenesis via the powerful pro-coagulant thrombin, which acts in the final common pathway bypassing the effects of ADP on coagulation cascade. The results could have been different if platelet mapping algorithm was used, which measures effects of individual platelet agonists like ADP and arachidonic acid [23]. The consistent lack of correlation between platelet reactivity and thrombus area suggest that point of care measure of platelet reactivity does not reflect whole blood thrombogenicity which is a complex pathophysiology involving other factors in addition to platelets. Our results may in part explain the negative results of the GRAVITAS and ARCTIC studies using VerifyNow® [24,25].

Our findings provide additional mechanistic support for the proposal that ‘perpetuation and propagation of diabetic thrombus’, with a pathophysiological cycle of “thrombus begets thrombus”, is responsible for increased recurrent acute coronary events in T2DM patients [26]. It is known that patients with T2DM have ‘vulnerable blood’ which is responsible for more major adverse cardiovascular events after NSTEMI-ACS [27]. There are no published studies of differences in the visco-elastic properties and fibrin structure of thrombus in patients with and without T2DM after NSTEMI-ACS. It is possible that loosely bound diabetic thrombus which is higher in quantity may also embolise easily to distal arteries. Nevertheless, once formed, the diabetic thrombus was slower to undergo clot retraction (early autolysis) and along with the well known defects in fibrinolysis (late autolysis), these findings support the view that ‘perpetuation and propagation of diabetic thrombus’ is responsible for recurrent acute coronary events [28]. Thus our findings, in a small cohort, are novel, hypothesis generating and require further validation.

By using whole blood testing we have reported a comprehensive evaluation of thrombotic potential in patients with T2DM. These findings provide mechanistic insight into the higher risk of thrombotic events seen in T2DM patients in contemporary studies [29,30]. The differences seen in the ultrastructure and mechanistic properties of thrombus provide a focus for further studies that may lead to novel pharmacological treatments targeting the fibrin pathway.

There are significant limitations in our study. Our cohort is small and the generalisability of our findings needs to be confirmed. However, our subjects were carefully selected to maintain homogeneity of patient characteristics. Our findings are descriptive in nature and therefore need to be confirmed by experimental and interventional studies. We studied aspirin and clopidogrel in our study and the effects of newer antiplatelet agents on thrombus structure and viscoelastic property in T2DM remain unknown.

Conclusion

In patients with T2DM after NSTEMI-ACS, we found increased thrombus which was slower to undergo autolysis, with altered fibrin

architecture. The combination of increased quantity and persistence of thrombus may explain the “vulnerable blood” and higher thromboembolic events in T2DM after NSTEMI-ACS. The differences seen in the ultrastructure and mechanistic properties of thrombus provide a focus for further studies that may lead to novel pharmacological treatments in the diabetic patients.

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Conflicts of Interest Statement

1. Dr Girish N Viswanathan: None
2. Prof Sally M Marshall: None
3. Dr Karthik Balasubramaniam: None
4. Prof Juan J Badimon: None
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GNV, SMM and AGZ conceptualised and conducted the study, analysed and interpreted the data and wrote the manuscript. KB edited the manuscript and JJB interpreted the data and edited the manuscript. All authors approve the final version of the manuscript.

Prof Azfar Zaman, is the guarantor of this work. He certifies that he had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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